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Folate bioavailability: UK Food Standards Agency workshop report

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The UK Food Standards Agency convened a group of expert scientists to review current research investigating folate bioavailability. The workshop aimed to overview current research and establish priorities for future research. Discrepancies were observed in the evidence base for folate bioavailability, especially with regard to the relative bioavailability of natural folates compared with folic acid. A substantial body of evidence shows folic acid to have superior bioavailability relative to food folates; however, the exact relative bioavailability still needs to be determined, and in particular with regard to mixed diets. The bioavailability of folate in a mixed diet is probably not a weighted average of that in the various foods consumed; thus the workshop considered that assessment of folate bioavailability of whole diets should be a high priority for future research.

Folate bioavailability: Folic acid supplementation: Food Standards Agency workshop: Nutrition research

The UK Food Standards Agency (FSA) convened a workshop on 27 January 2003 on folate bioavailability. The results from recently completed studies were presented, both FSA and non-FSA funded, and the workshop was chaired by Professor Jess Gregory, University of Florida, USA. The aim of the workshop was to determine where this work has taken us and where further work should be concentrated, as well as acting as a vehicle for dissemination. The research recommendations will feed into the future direction of FSA-funded nutrition research, and may also be of value in guiding other funders.

Background

Folate is a generic term for a B-group vitamin found widely in foodstuffs. There is a large family of naturally occurring folates; mostly reduced tetrahydropteroylglutamates, often in polyglutamyl form and usually one-carbon substituted. Folate vitamers in foods occur mainly as reduced methyl- and formyl-tetrahydropteroylpolyglutamates (Perry, 1971; Scott & Weir, 1976). Folic acid (pteroylmonoglutamic acid) is the synthetic form used in supplements and food fortification. Folates function coenzymically in the transfer and processing of one-carbon units and play an important role in nucleotide synthesis, methylation, and gene expression (Shane, 1995). Examples are: (i) the synthesis of thymidine, which is essential for the *de novo* construction or repair of DNA; (ii) the remethylation of plasma homocysteine to methionine; (iii) the 'site-specific' methylation of the cytosine base in DNA, which regulates gene expression. Folates play a key role in disease prevention, with low folate status associated with increased risk for cardiovascular disease, neural tube defects (NTD), colorectal cancer and dementia (Medical Research Council Vitamin Study Research Group, 1991; Boushey *et al.* 1995; Blount *et al.* 1997).

The issue of the bioavailability of food folates became apparent with the publication of the classic paper by Tamura & Stokstad (1973). Although aspects of their methodology have been criticized, this study showed that

Abbreviations: FSA, UK Food Standards Agency; MTHF, methylenetetrahydrofolate; MTHFR, methylenetetrahydrofolate reductase; NTD, neural tube defects; tHcy, total homocysteine; THF, tetrahydrofolate.

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the bioavailability of folate in a wide variety of foods was incomplete and highly variable. Bioavailability can be a major determinant of nutritional status when folate intakes are in the marginal range.

Bioavailability is defined in many ways, most of which focus on the efficiency of intestinal absorption. Post-absorptive metabolism and excretion processes can affect bioavailability in certain contexts, but this is mainly relevant to intakes of folic acid (or other supplements) at doses that exceed the requirement and the metabolic capacity. Recent terms such as bioefficacy and bioconversion (van Lieshout *et al.* 2003) extend the concept of bioavailability. Regardless of definition though, bioavailability depends on many factors and is not a constant (Bronner, 1993).

The bioavailability of folic acid (both supplemental and in fortified foods) is almost always substantially higher than the net bioavailability of naturally occurring food folate. Factors thought to affect folate bioavailability include: incomplete release from plant cellular structure (van het Hof *et al.* 1999; Castenmiller *et al.* 2000); entrapment in the food matrix during digestion (Pfeiffer *et al.* 1997); instability during digestion (for example, tetrahydrofolate (THF); Seyoum & Selhub, 1998); partial inhibition of deconjugation by other dietary constituents (especially organic acids) (Bhandari & Gregory, 1992; Wei *et al.* 1996; Wei & Gregory, 1998); other dietary constituents increasing folate stability during digestion (for example, folate-binding proteins; Jones & Nixon, 2002); possibly other constituents such as ascorbate, or other reducing agents. Whether the degree of conjugation of polyglutamyl folates affects bioavailability remains unclear in view of conflicting data (Gregory, 1997).

Genetic factors have also been suggested to affect folate bioavailability, such as: a history of NTD-affected pregnancy (Davis *et al.* 1995; Neuhouser *et al.* 1998; Boddie *et al.* 2000); or the presence of a glutamate carboxypeptidase II single nucleotide polymorphism (Devlin *et al.* 2000), although this was not confirmed more recently (Lievers *et al.* 2002; Vargas-Martinez *et al.* 2002).

Determination of folate status

Serum and plasma folate concentrations are a responsive indicator of folate status and are commonly used in acute studies. Erythrocyte folate concentrations are a less responsive indicator, but are considered to be the best index of long-term status. The lifespan of the erythrocyte is 120 d, and folates are retained in the erythrocyte for the duration of its life; thus, less than 1 % of circulating erythrocytes are replaced daily (Gregory, 2001). Intervention studies, therefore, need a minimum duration of 3–4 months when assessing this index (Ward *et al.* 1997).

Plasma total homocysteine (tHcy) concentrations are also used as an indicator of folate status; elevated levels of tHcy are considered to be a risk factor for cardiovascular disease (Homocysteine Studies Collaboration, 2002) and NTD (Mills *et al.* 1995). The remethylation of homocysteine to methionine by methylenetetrahydrofolate (MTHF) reductase (MTHFR) is dependent on an adequate supply of folate; thus, low folate status results in elevated tHcy concentrations. Several other B vitamins are also required for the remethylation of homocysteine: cobalamin (B_{12}), vitamin B_6 and riboflavin (B_2), B_6 (McKinley *et al.* 2001) and B_{12} nutritional status (Quinlivan *et al.* 2002) may also affect tHcy concentrations.

Other factors as well as B vitamins may also affect tHcy concentrations; for example, alcohol, physical activity, dietary fibre (Mennen *et al.* 2002) and coffee (Verhoef *et al.* 2002). Furthermore, the modification to dietary patterns has been suggested to be more effective than increasing folate-rich foods alone in lowering tHcy (Appel *et al.* 2000). The C677T MTHFR polymorphism also influences tHcy concentrations. Its prevalence is related to ethnicity: the homozygous TT genotype is about 10 % in Caucasians (though it may be population-dependent, and is about 20 % or more in some Italian and US Hispanics) and only a few percent in Africans and Afro-Americans (Botto & Yang, 2000). The homozygous form (TT) is associated with elevated tHcy levels of typically 25 % (Engbersen *et al.* 1995).

The studies presented as part of this workshop investigated the efficacy of different folates on folate status; these included chronic feeding trials and more acute stable isotopic studies.

Chronic feeding studies

Professor Helene McNulty presented the results from an FSA-funded project comprising several studies examining the biological responses in healthy subjects to intervention with food folates or folic acid, as well as determining food folate concentrations. The main findings from the analysis of food folates and the effects of cooking on folate retention in foods (Mckillop *et al.* 2002) were:

- The boiling, but not steaming, of green vegetables (broccoli and spinach) resulted in significant losses (>50 %) of folates from the vegetables into the water. Boiling potatoes or grilling beef did not significantly affect their folate content.
- By using a tri-enzyme predigest (protease, amylase and conjugase) in the determination of food folate content (composite meals, spinach, broccoli, potato and beef), significantly higher values (1·3- to 6-fold) than the published values were obtained (Pentieva *et al.* 2002).

In all intervention trials, any homocysteine-lowering due to vitamin B_6 or B_{12} deficiency was corrected by pre-treatment with physiological doses of these vitamins before commencing the folate intervention. Subjects were healthy males aged 18–45 years who were not consumers of B-vitamin supplements or fortified foods, and not homozygous for the thermolabile (C677T) variant of the MTHFR gene. The main findings from the intervention trials were:

- Subjects (*n* 20) were administered with incremental doses of folic acid (100, 150, 200 and 400 μg/d) for a total period of 32 weeks. Intervention resulted in significant incremental increases in serum folate and corresponding significant decreases in plasma tHcy, with the maximum reduction in tHcy observed in response

to 200 μg/d; no further significant lowering was observed in response to 400 μg/d administered for 14 weeks, despite significant increases in serum folate concentration (Ward *et al*. 2002)

- Subjects (*n* 24) received 200 μg folic acid/d for 12 weeks as either a supplement or as fortified bread. Both treatments resulted in equivalent reductions in tHcy and increases in serum folate concentrations, demonstrating the high bioavailability of folic acid in fortified bread.
- Subjects (*n* 45) were supplemented for 30 d with one of four treatments: folic acid (200 μg/d); spinach folate extract (200 μg/d); yeast folate extract (200 μg/d); or placebo (McKillop *et al*. 2003). Folic acid supplementation raised serum folate levels and reduced tHcy levels to a larger extent than the other treatments; relative bioavailability was calculated as 31 % for spinach and 53 % for yeast.
- Subjects (*n* 73) were supplemented for 30 d with 0·2 mg folate/d as either: folic acid (*n* 18); spinach drink (*n* 12) or meal (*n* 6); yeast drink (*n* 13) or meal (*n* 6); or placebo (*n* 18). All treatments raised folate status as determined by serum folate and tHcy concentrations; however, folic acid supplementation was more effective than either spinach or yeast, which showed overall relative bioavailabilities of 33–44 % (spinach) and 45–62 % (yeast) (MPA Hannon-Fletcher, NC Armstrong, JM Scott, M Ward, JJ Strain, K Pentieva, AA Dunn, AM Molloy, M Scullion and H McNulty, unpublished results).

In general, there was no evidence that foods that have a greater percentage of folate in the conjugated form (for example, yeast, which contains virtually all polyglutamates) were less bioavailable than those with less (for example, spinach, which contains about 50 % polyglutamates; egg, which contains 0 % polyglutamates). The food matrix did not appear to exert an effect on folate bioavailability, in that similar results were found whether or not the food was administered with the food matrix intact. Estimations of relative folate bioavailability tended to be lower when food folates were provided as a meal compared with as a drink, suggesting that the presence of other (non-folate) foods ingested at the same time adversely affects the bioavailability of food folate.

Professor Pierpaolo Mastroiacovo presented results from a 13-week placebo-controlled randomized trial (P Mastroiacovo, E Carnovale, A Turrini, L Mistura, S Ruggeri, E Camilli, R Ricci, O Genovese, B Zappacosta, S Persichilli, A Minucci, G Andria, A Boninconti, unpublished results) investigating in 149 free-living subjects (18–60 years, mean 40·4 years) with moderate hyperhomocysteine (mean 12·8 μmol/l) using three strategies for increasing daily folate: dietary counselling to add around 200 μg natural folate/d; 200 μg folic acid/d; 200 μg 5-MTHF/d. The primary outcome was the effect on tHcy concentrations; the secondary outcome was the effect on erythrocyte folate concentrations. Randomization was performed by MTHFR C677T genotype stratum. The main findings from the study were:

- The mean total daily intake of folate in the four randomized arms was: 350 μg/d in the dietary-counselling group; 420 μg/d in the 5-MTHF group; 403 μg/d in the folic acid group; 220 μg/d in the placebo group. The dietary assessment of subjects was conducted using a food frequency questionnaire; in the diet group the vegetables and fruits that contributed most to folate intake were Swiss chard, French beans, artichoke, spinach, asparagus and broccoli (among vegetables) and orange, orange juices, kiwi and mandarin (among fruits).
- The absolute and relative mean variations of plasma tHcy (geometric mean adjusted on starting values) and the ratio *v.* the placebo group were respectively:

  (a) enriched diet: −3·2 μmol, −23·0 %, ratio 0·79;
  (b) 5-MTHF: −3·1 μmol, −22·3 %, ratio 0·80;
  (c) folic acid: −3·3 μmol, −24·1 %, ratio 0·77;
  (d) placebo: −0·9 μmol, −7·5 %, ratio 1·00 (reference).

- The subgroup analysis by MTHFR C677T genotype did not show relevant differences among the three genotypes (TT, CT, CC) or among the homozygous TT individuals and the others (CT, CC).
- The mean erythrocyte folate levels increased significantly during the 13 weeks in the three treated groups, but not in the placebo group. There was a statistically significant difference between the treated groups (marginal for the 5-MTHF group) and placebo group. Mean erythrocyte folate levels were highest after supplementation with folic acid, but not significantly higher than the levels observed in the dietary-counselling group.

Increasing natural folate intake by dietary counselling decreased tHcy concentrations to an extent similar to that observed with 200 μg folic acid/d or with 200 μg 5-MTHF/d. The effect seems similar to that observed with higher doses of folic acid (Homocysteine Lowering Trialists' Collaboration, 1998), which may be due to the high subject baseline levels of tHcy; an important determinant of the size of the tHcy-lowering response. The effect obtained with an enriched diet of natural folate is clinically relevant since one out of three individuals had a decrease of one-quarter of his starting tHcy. Although subjects with the common polymorphism MTHFR C677T had higher initial tHcy levels, this did not influence the treatment effect. If these results are confirmed by other studies, population dietary modification may be an effective strategy to decrease tHcy and possibly reduce the risk of cardiovascular disease in the population.

Dr Ian McDowell presented results from a study (Moat *et al.* 2003) that measured B-vitamin status and plasma tHcy in 126 healthy subjects aged 20–63 years (forty-two CC, forty-two CT and forty-two TT *MTHFR* genotypes) at baseline and following three sequential interventions of 4 months each: placebo plus natural diet; supplement of 400 μg folic acid/d plus natural diet; increased dietary folate (including folic acid-fortified foods) to 400 μg/d. This study was primarily designed to assess the effect on homocysteine of dietary or low-dose folate supplements in relation to MTHFR genotype combined with an assessment of its effect on vascular endothelial function (Pullin *et al.*

2001). Riboflavin status was evaluated as an additional study. The main findings from this study were:

- Plasma tHcy was significantly higher in those with the TT genotype and responded to increased folate intake with dietary or supplemental folate to similar extents. Subjects with the TT genotype were particularly responsive to enhanced folate intake (Ashfield-Watt *et al.* 2002).
- At baseline and following nutritional interventions, lower riboflavin status was associated with increased plasma tHcy concentrations. tHcy was 2·6 μmol/l higher in the lowest plasma riboflavin quartile compared with the highest ($P<0·02$) and was 4·2 μmol/l higher in the highest erythrocyte glutathione reductase activation coefficient quartile compared with the lowest ($P<0·001$). This effect was not restricted to those with the T allele (observed in TT and CT, but not CC).
- There was a high prevalence of low riboflavin status in the study population and folic acid supplementation appeared to exacerbate this. This suggests that folic acid supplementation alone may increase the rate of turnover of flavins, thereby exacerbating any tendency to riboflavin deficiency.

Folate and riboflavin appear to interact to lower plasma tHcy. The effect of folate is most pronounced in those with the TT gentoype but that of riboflavin may be unrelated to the MTHFR genotype. However, other studies (Jacques *et al.* 2002; McNulty *et al.* 2002) report that any effect of riboflavin status is confined to the TT genotype and Yamada *et al.* (2001) observed that the TT variant releases its riboflavin-derived cofactor FAD much faster than the wild-type enzyme.

Alida Melse-Boonstra presented results from a series of studies undertaken at Wageningen Centre for Food Sciences, The Netherlands. The main studies were:

A double-blind placebo-controlled dose-finding trial to determine the lowest folic acid dose that lowers tHcy concentrations adequately in healthy older adults (316 men and women, 50–75 years). Subjects were randomly assigned to one of seven treatment groups: 50, 100, 200, 400, 600 and 800 μg folic acid, or placebo daily for 12 weeks. Groups were stratified for baseline tHcy concentrations. A maximal reduction in tHcy concentration was observed at 0·4 mg/d; 0·2 mg/d resulted in a reduction in tHcy of 77 % of that observed with 0·4 mg/d (van Oort *et al*. 2003).

Subjects (180 men and women, 50–75 years) were randomized to one of three treatment groups receiving daily either 323 nmol (about 200 μg/d) monoglutamyl folic acid, 262 nmol (about 285 μg/d) heptaglutamyl folic acid or a placebo capsule. Serum and erythrocyte folate concentrations, and tHcy concentrations, were measured after 2 and 12 weeks of intervention. The relative bioavailability of heptaglutamyl folic acid was 64 (95 % CI 52, 75) % based on serum folate and 68 (95 % CI 51, 84) % based on erythrocyte folate. The relative bioefficacy, that is the ability of folic acid to lower homocysteine concentrations, of heptaglutamyl folic acid was found to be 106 (95 % CI 77, 134) %.

These studies show that in older adults, daily supplementation with folic acid effectively lowers tHcy concentrations, with no further reductions being observed at doses above 0·4 mg/d. Also, the polyglutamyl form of folic acid had lower bioavailability than the monoglutamyl form.

Stable isotopic studies

The advantage of isotopic labelling studies is their specificity; however, a disadvantage can be sensitivity, due to the dose of a stable isotopically labelled folate tracer required to yield measurable enrichment of blood or urine folates. This limitation can be overcome, in part, by using protocols involving repeated small doses.

Alida Melse-Boonstra presented results from a pilot study using a newly developed dual-label stable-isotope protocol: three subjects (aged 20–30 years) took 200 nmol (about 90 μg/d) [$^{13}C_{11}$]monoglutamyl folic acid and 200 nmol (about 190 μg/d) [$^{13}C_6$]hexaglutamyl folic acid daily for 10 d in the form of a capsule. Fasting blood samples were collected at days 3, 6 and 10 after supplement administration and analysed for concentrations of [$^{13}C_{11}$]folate, [$^{13}C_6$]folate and unlabelled folate. This was done by a liquid chromatography-MS–MS method. The relative bioavailability of hexaglutamyl folic acid was calculated by taking the ratio of plasma enrichments of [$^{13}C_6$]folate and [$^{13}C_{11}$]folate at 10 d.

Results revealed a bioavailability of 70 (range 66–72) % of hexaglutamyl folic acid as compared with the monoglutamyl form.

The pilot study has been repeated in twenty subjects (men and women, aged 20–50 years) and preliminary data analysis indicates comparable results with the pilot study. This confirms the findings from the chronic feeding study and suggests that folate bioavailability can be studied successfully with this dual-label stable-isotope protocol with multiple dosing.

Paul Finglas presented the results from an FSA-funded project (Wright *et al*. 2003) involving acute stable-isotope studies using a direct liquid chromatography–MS technique, and intrinsic stable-isotope-labelled spinach folates. ^{13}C-labelled oral test folate isolates (folic acid, *n* 14; 5-formyltetrahydrofolic acid, *n* 16) and ^{15}N-labelled spinach folates (mainly 5-methylTHF and 5-formylTHF, *n* 14) were administered to volunteers, and the plasma folate response (both total and labelled) was analysed by HPLC and liquid chromatography–MS over the following 8 h. The main findings from these studies were:

There was a marked difference in the absorption and metabolism of folic acid *v.* the natural folates: the rate of appearance in plasma was slower and the total plasma folate response was less following administration of folic acid. Also, administration of all forms resulted in an increase in non-test dose unlabelled plasma folate, and this was less so for folic acid.

Mathematical modelling of the data suggested that the liver sequesters a proportion of all newly absorbed folate (the first-pass effect), which was significantly greater following the oral administration of folic acid (about 70 %) than for natural folates (about 50 %, or lower).

There was a strong correlation (r 0·94; $P<0·001$) between the labelled plasma response to an oral dose of labelled folic acid and fasting baseline plasma folate concentration, which was not observed for the natural folates. It was suggested, therefore, that folic acid may not be reduced and methylated at the mucosal level but appears unchanged in the hepatic portal vein where it is removed more efficiently than any 5-methylTHF derived from other reduced folates (5-formylTHF or spinach folate). It was suggested that physiological doses of folic acid transferred from mucosal cells to the hepatic portal vein are removed in their entirety (i.e. 100 %) on the first-pass to the liver, and all the subsequent labelled 5-methylTHF plasma response is entirely a function of enterohepatic recirculation.

It appears, therefore, that folic acid is metabolized differentially by the liver as compared with natural folates; more of the folic acid form was taken up by the liver from the portal vein suggesting the liver to be the primary site for its subsequent reduction and methylation. This has implications for acute methods of determining relative folate bioavailability that rely upon measuring plasma folate levels in response to the oral administration of folates relative to that of a 'reference dose' of folic acid. However, whether folic acid is methylated and reduced by the liver still requires further investigation.

Discussion

There are discrepancies in the evidence base for folate bioavailability, especially with regard to the relative bioavailability of natural folates compared with folic acid. A substantial body of evidence shows folic acid to have superior bioavailability relative to food folates; however, the exact relative bioavailability still needs to be determined, and in particular with regard to mixed diets. The use of dietary folate equivalents, in principle, is a good way to express dietary recommendations because it adjusts for the apparent difference in bioavailability between dietary folate and added folic acid. However, the reliability of this approach is questionable because of the uncertainties in the relative bioavailability of each. The bioavailability of folate in a mixed diet is probably not a weighted average of that in the various foods consumed. Thus:

the screening of various individual foods should be a low priority except in mechanistic studies or to verify efficacy;

assessment of folate bioavailability in food classes is a higher priority;

assessment of whole diets is a high priority (for example, Sauberlich *et al.* 1987).

With regard to tHcy lowering, it is particularly important to assess the impact of the whole diet, as it is affected by a number of dietary factors.

An added complication to the assessment of folate intake is that the accuracy of food composition tables is variable. This was highlighted at the workshop with the application of improved methodologies for food folate determination. The FSA is currently assessing the impact of this on its published food composition tables.

Priorities and research recommendations identified at the workshop

The workshop recommended that:

there should be an increased focus on whole diets;

mechanisms affecting bioavailability are a priority for research;

improved methods are needed for the assessment of bioavailability of naturally occurring folates.

Participants

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